Mapping the testicular interstitial fluid proteome from normal rats

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Abstract

Communication between the testicular somatic- (Sertoli, Leydig, peritubular myoid, macrophage) and germ- cell types is essential for sperm production (spermatogenesis), but the communicating factors are poorly understood. We reasoned that identification of proteins in the testicular interstitial fluid (TIF) that bathes these cells could provide a new means to explore spermatogenic function. The aim of this study was to map the proteome of TIF from normal adult rats.

Low-abundance proteins in TIF were enriched using Proteominer beads, and identified by MALDI tandem mass spectrometry, recognising 276 proteins. Comparison with proteomic and genomic databases showed these proteins originated from germ cells, somatic cells (Sertoli, peritubular myoid, Leydig) and blood plasma. *In silico* analysis revealed homologues of >80% TIF proteins in the human plasma proteome, suggesting ready exchange between these fluids. Only 36% of TIF proteins were common with seminiferous tubule fluid that transports mature spermatids to the epididymis, indicating these two fluids

are quite different. This TIF proteome provides an important new resource for the study of intercellular communication in the testis.

Significance

The testis is composed of multiple cell types that co-operate to produce sperm. Elucidating how these cells communicate is important for understanding normal sperm production and how it is disturbed in infertility. An important goal for new strategies to better diagnose male infertility is to find proteins that reflect the ability of the testes to produce mature sperm. This study represents a first step towards this goal; by defining the proteome of testicular interstitial fluid (or TIF) in adult rats with normal sperm production, and comparing it to other relevant datasets. We describe 276 proteins that were detected in TIF. We show that TIF is quite different to seminiferous tubule fluid, and importantly, that many TIF proteins are present in blood plasma and could therefore potentially be detected by blood tests. This TIF proteome will serve as an important resource for research into sperm production and conditions of infertility.

Introduction

Sperm production (spermatogenesis) occurs within the seminiferous tubules, where germ cells develop in association with, and supported by, the somatic Sertoli cells (Fig 1). Outside of the tubules is the interstitium, containing androgen-secreting Leydig cells, macrophages, blood and lymphatic vessels. Tight junctions between Sertoli cells create a

blood-testis barrier, which sequesters advanced germ cells in a unique milieu distinct from the vascular environment (Fig 1). The immature germ cells (spermatogonia) and Sertoli cells lie outside this barrier with free access to the interstitium and vasculature. More advanced germ cells and developing sperm reside above the barrier and are thus separated from the interstitium and vasculature.

Communication between testicular cells is essential for spermatogenesis and steroidogenesis. For example, peritubular myoid cells (PTMCs) (Fig 1) surround the seminiferous tubules and maintain Sertoli cell function, and sperm production [1]. Sertoli cells regulate PTMC function (e.g. [2]) and Leydig cell steroidogenesis (e.g. [3]), and testicular macrophages modulate Leydig cell function [4]. These cells are not all in physical contact yet are highly secretory, therefore communication occurs via soluble factors secreted into testicular fluids, including testicular interstitial fluid (TIF). Proteins from various testicular cells have been identified in TIF [5], including Sertoli- [5], Leydig- [6], and germ cells [7]. Both the volume of TIF and the concentrations of some TIF proteins are responsive to changes in endocrine hormones which drive spermatogenesis [8, 9], and to alterations in the local environment, including changes in germ cells [10]. Therefore the composition of TIF can reflect the status of spermatogenesis.

Despite considerable effort to find molecular markers of spermatogenesis in readily-accessible biological fluids (plasma, seminal plasma) (e.g. [5, 7, 11-13]), the diagnosis of men with infertility still relies on assessment of sperm number, motility and morphology in the ejaculate, and on testis volume and serum hormones ([14, 15]). These assessments give limited information when no sperm is present in the ejaculate (azoospermia), because they do not reliably inform about the extent of spermatogenesis in the testis. For example, they cannot

always distinguish between men with primary spermatogenic failure (non-obstructive azoospermia) and those with complete spermatogenesis but a physical blockage post-testis (obstructive azoospermia). Current approaches provide limited information about the potential for spermatogenic improvement with hormonal treatments, or on the presence of mature spermatids that could be recovered by testicular sperm extraction for assisted reproductive treatment [16-18]. For these applications, a testicular biopsy remains the definitive test, but requires specialist surgical skills and carries inherent risks [19]. The identification of proteins in testicular fluids and/or blood plasma that reflect spermatogenic function could therefore be of use in exploring mechanisms underlying infertility.

We hypothesise that TIF contains proteins that reflect the cellular functions involved in spermatogenesis. Surprisingly little attention has been given to the TIF proteome since several germ cell proteins in rat TIF were identified using 2D gels in the mid-90's [7]. We aimed to use more sensitive proteomic methods to map the proteome of TIF in normal adult rats as a first step towards future studies of models of animal and human infertility.

Experimental Procedures

TIF collection: All animal experimentation was approved by Monash Medical Centre Animal Ethics Committee. Untreated adult male outbred Sprague Dawley rats (n=3, 75–90 d, 400-500g) were sacrificed by CO_2 inhalation. Testes were immediately removed, cleaned of connective tissue, washed in ice-cold PBS pH7.4 containing Complete protease inhibitor cocktail (Roche, Castle Hill, Sydney) then patted dry on filter paper. A 2-4mm incision was made in the tunica at the distal end, and each testis was suspended in a 15ml tube above 20μl

PBS/inhibitors via a thin suture placed through the tunica at the other end. TIF was collected by percolation for ~16hr at 4C [9], then centrifuged (10,000g, 15min, 4°C) and the supernatant stored at -80°C prior to enrichment.

Enrichment: Protein enrichment of TIF was achieved using a ProteoMiner protein enrichment large capacity kit (BioRad, Hercules, CA, USA) according to the manufacturer's instructions, and low abundance proteins were reduced and alkylated with iodoacetamide and digested with trypsin as described [20].

Fractionation: Peptides (from ~400μg total protein) were fractionated by isoelectric focussing using an OFFGEL 3100 Fractionator (Agilent Technologies, CA, USA) with an Immobiline™ DryStrip, linear pH range 3-10, 24 cm (GE Healthcare, Uppsala, Sweden) [20]. Peptides were focused at a constant current of 50 mA at 25°C for 50,000 volt hours. After focusing, 24 fractions were collected and stored at -80°C.

Liquid Chromatography and MALDI-Mass Spectrometry (LC-MALDI-MS/MS): Each OFFGEL fraction was further fractionated by HPLC and robotically spotted to MALDI target plates, resulting in 9,216 spots analysed by MALDI-TOF. Full details, including MS/MS search parameters, are included as Supplementary Document 1. Only proteins with a stringent MASCOT score >50 were included for further analysis.

SDS-PAGE, Western blotting and Immunolocalization: Samples (~30μg/lane) were boiled in Tris-SDS sample buffers and applied to pre-cast 4-12% Criterion XT gels (BioRad). Gels were either stained with SyproRuby and imaged on a Fuji FLA5100 fluorescence laser scanner, or transferred to nitrocellulose using a Transblot Turbo-Western system (BioRad). Membranes were blocked with Odyssey blocking buffer (1:4 for 1hr at 22°C, Li-Cor,

Lincoln, NE, USA), then incubated overnight at 4°C with primary antibody (musashi-2, lumican, INSL-3, α-actinin; see Supplementary Table 1). The secondary antibodies were goat anti-rabbit IgG – Alexa Fluor 680 (1:5,000, Molecular Probes, Eugene, OR, USA) or biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories, Peterborough, UK). Antibody signals were imaged using an Odyssey detection system (Li-Cor).

All immunohistochemical procedures were carried out as described [21, 22]; primary antibody details are shown in Supplementary Table 1. Antibody signals were visualized with a confocal microscope (FV-300, Olympus, Shinjuku, Japan), or by light microscopy following incubation with streptavidin-conjugated HRP (1:1,000 dilution, Vector) and 3,3'-diaminobenzidine with haematoxylin counterstaining. For western blots and immunohistochemistry, specificity was monitored by the substitution of the primary antibody with an equivalent dilution of affinity-purified non-immune rabbit IgG.

Bioinformatic analyses: The functional annotation clustering tool within the DAVID Bioinformatics Resource (v6.7) was used to group proteins into related biological processes (http://david.abcc.ncifcrf.gov/) [23].

Results

1. Identification of adult rat TIF proteins

The volume of TIF recovered per adult rat testis was ~85µl, with a protein concentration of 39.8mg/ml. After Proteominer Bead-enrichment, the pooled bound fraction (2-3% of total protein) showed an increase in low-abundance proteins by SDS-PAGE (Fig

2A), and was digested with trypsin prior to isoelectric focusing. LC-MALDI-MS/MS analysis identified 40-60 proteins per fraction over the pH range 3.0-7.5, with only 5-10 proteins/fraction in the basic fractions pH 7.8 – 9.9 (Supplementary Figure 1).

A total of 276 proteins with MOWSE scores >50 were identified. The top 30 are shown in Table 1, the entire list is in Supplementary Table 2 and detailed protein and peptide sequence data are in Supplementary Table 3. The proteome contained well-known TIF proteins such as Insl3 secreted by Leydig cells [6] (rank #262, Supplementary Table 2), transferrin (rank#15), well known Sertoli cell proteins alpha2 macroglobulin (rank#24) and clusterin (rank #43) [24-26], and Pebp1 (rank #106) previously described in rat TIF [7] and expressed in Sertoli and germ cells. TIF also contained diverse proteins including RNA splicing / binding enzymes (Isyna1, Gstm1, Aldh2, Sod3), extracellular matrix proteins (Lum, Col1a2, Efemp1) and serum factors involved in complement activation (C8b, C9, SerpinG1) and the inflammatory response (A2m, Ass1, Masp1).

2. *In silico* analysis of TIF proteins

To build a more complete picture of normal rat TIF, we performed *in silico* analysis to identify the cellular source(s) of TIF proteins, and compared the TIF proteome to other testicular fluid proteomes.

Comparison to other testicular fluid proteomes

The TIF proteome was compared with three relevant proteomes; 1) *seminiferous tubule fluid (STF)* proteome from adult ram and rat containing 1051 proteins [27]; STF is fluid from the tubule lumen (Fig 1) which carries mature spermatids via the rete testis into the epididymis, and is largely formed by Sertoli and germ cell secretions, with limited access to

the vascular and interstitial compartments. 2) *Human PTMC* proteome containing 1785 proteins from cultured cells, including 262 proteins secreted by PTMCs [28]. 3) *Human plasma* proteome via the Human Plasma Protein Database (http://www.plasmaproteomedatabase.org) [29]. Comparisons were according to gene annotation [27].

Several interesting points were noted. TIF was only 23% similar to rat or ram STF respectively (Supplementary Table 2). When the STF proteomes were merged to form a combined STF protein list, the similarity to the rat TIF proteome increased to 36% (Fig 2B). 41% of TIF proteins were found in the PTMC proteome (Supplementary Table 2), consistent with the localisation of these cells in the interstitial compartment (Fig 1). The proportion of TIF proteins not represented in either STF or peritubular myoid cell data-sets was 39.0% (n=104 proteins). A search for homologues of rat TIF proteins in the human plasma proteome revealed >80% proteins in common (Supplementary Table 2), supportive of a ready exchange between these two fluids in the testis, and also indicative that TIF proteins could come from extra-testicular sources.

Comparison to microarray data

To determine the potential contribution of seminiferous epithelial cell types to the TIF proteome, we conducted *in silico* comparisons with mRNA expression data, as individual cell proteomes are unavailable. We assessed microarray data from isolated Sertoli cells and various germ cell types from mouse and rat [30] and a similar purified cell dataset from rats [31]. Both studies grouped germ cells into spermatogonia, pachytene spermatocytes, and round spermatids. As most genes were present in one or more of these cell groupings

(Supplementary Table 2), semi-quantitative criteria were designed to highlight genes that were expressed in one cell type i) >10fold, or ii) >4-fold, or iii) between 2-4 fold higher than any other cell type. Genes with differences <2 fold were considered to be equally expressed (see Table 1 and Supplementary Table 2).

Using these criteria, several patterns became apparent. 34% of TIF proteins showed preferential mRNA expression in Sertoli cells and/or spermatogonia (Supplementary Table 2: e.g. #90; *Ecm1*, # 151; *Ctsb*, #269, *Sod3*) whereas 28% of TIF proteins were expressed in all seminiferous epithelial cell types (e.g. #66; *Sptan1*, #92; *Eef1a1*; #217; *Phrf1)*. Overall, 178 (64%) of TIF proteins showed mRNA expression in Sertoli cells and/or spermatogonia, and could thus potentially reflect spermatogenic function (Supplementary Table 2). Only 3.6% were preferentially expressed in pachytene spermatocytes and/or round spermatids which reside inside the blood testis barrier (e.g. #30; *Hrg*, #197; *Ubqln1*, #235; *Spata24*, # 238; *Dctn2*), and of this group 3 proteins (*Itih3, Hrg, Mapt*) showed mRNA expression exclusively restricted to spermatocytes and spermatids. Homologues of these three proteins were also present in human plasma.

24% of TIF proteins were not expressed in any epithelial cell type and thus may be derived from other testicular cells (see below) or from extra-testicular sources. The final 11% of proteins had no data available by microarray, and usually corresponded to known plasma proteins or in some cases to genes below the detection limit of the arrays. Examples of TIF proteins in which the mRNA expression was highly enriched in specific cell types (>10fold) included: #28; *Itih3* #228; *Mapt* (in round spermatids), #56; *Efemp1*, #71; *Lum*, #81; *Col3a1*, #157; *Ogn*, #176; *Prrc1*, #259; *Fcn1* (in spermatogonia), and #136, *Pah* (Sertoli cells).

Comparison to Leydig cell microarray

We sought to determine which TIF proteins could be derived from interstitial Leydig cells (Fig 1) using microarray data from the rat [32, 33] and mouse [34]. However, unlike the Sertoli and germ cell data where all microarray genes were searchable [30, 31], the available Leydig cell microarray data was processed and only records genes judged by the authors to be Leydig cell-specific [32-34]. Hence a negative finding in these columns (Table 1 and Supplementary Table 2) indicates that the gene is not Leydig cell specific, but it could still be expressed by Leydig cells. On this basis, 12 TIF proteins exhibited Leydig cell specificity based on rat microarray data, of which 7 were in agreement between the two rat studies [32, 33], these being *Hpx*, *Pah*, *Ces1*, *Ass1*, *Hmgcs2*, *Hp*, and *Insl3* (Supplementary Table 2). A further 67 TIF proteins were expressed by mouse Leydig cells (Supplementary Table 2), suggesting that at least 28% of the TIF proteome could be Leydig cell-derived, however this percentage could be higher noting the above caveat about data specificity. Only 2 genes (*Ass1*, *Hmgcs2)* were in agreement between all three data sources.

Gene ontology analysis of rat TIF

GO analysis of the biological processes enriched in rat TIF found 11 clusters with enrichment scores between 11.8 and 2.9, after applying a cut-off FDR score > 0.01 (Table 2). Processes relating to ‘coagulation’, ‘acute inflammatory response’, ‘assembly of complex proteins’ and ‘nutritional response’ were strongly over-represented in TIF (Table 2, Supplementary Table 4). Additional processes of interest were ‘hormone signaling’, ‘regulation of cellular protein metabolism’, and ‘cell cycle’.

3. Validation of selected TIF proteins

Immunolocalization and western blotting were used to validate the expression of four TIF proteins representative of different cellular functions. Lumican is an extracellular matrix (ECM) protein of the small leucine-rich proteoglycan family important in collagen organization, epithelial cell migration and tissue repair [35] and is secreted by PTMCs [28]. Immunolocalization showed weak cytoplasmic staining in spermatogonia and Sertoli cells, punctate staining around elongated spermatid heads and strong staining in interstitial cells. A single specific band at ~27kDa was detected for lumican in TIF (Figure 3A) which is lower than native lumican (~38kDa), and suggests a possible proteolytic event in rat TIF lumican that is supported by the C-terminal location of all 4 peptides detected by mass spectrometry (Supp Table 3, protein #81).. Musashi 2 (Msh2) is a nuclear DNA mismatch repair protein for DNA replication and recombination, known to be important in the testis and localized in spermatogonia and early spermatocytes [36]. This staining pattern was confirmed, with additional punctate staining in interstitial cells (Figure 3B). Western blotting showed a single specific band at ~95kDa in TIF (Figure 3B). Insulin-like 3 (Insl3) is a small peptide hormone secreted by Leydig cells [6]. Insl3 immunostaining was detectable in the interstitium (Figure 3D) although western blotting was unsuccessful, potentially due to its small size (6-15kDa). Alpha-actinin-4 (Actn4) is a ubiquitously expressed cytoskeletal microtubule anchoring protein but also functions as a nuclear steroid receptor co-activator [37]. Strong immunostaining for Actn4 was observed in blood vessels within the interstitium, with a band of ~100kDa in TIF (Figure 3E).

Discussion

This study is the first to define the protein composition of TIF using mass spectrometry. The results provide information on 276 proteins in TIF from normal adult rats. We show that rat TIF is comprised of proteins that are expressed in Sertoli cells and germ cells of the seminiferous tubules, as well as proteins that are likely to be produced by the PTMCs and the steroidogenic Leydig cells. Many TIF proteins (64%) showed mRNA expression in Sertoli cells and/or spermatogonia, suggesting the TIF proteome could reflect the function of these cells. A comparative analysis of the TIF partial proteome with previously published proteomic analyses of STF reveals that the proteomic composition of the two fluids varies considerably. Only 36% of proteins secreted into the adluminal compartment of the testis and detected in STF were detected in TIF, likely highlighting the directional secretion ability of the seminiferous epithelium, and the function of Sertoli cell tight junctions that form a major part of the blood-testis barrier [38-40]. It was also noteworthy that a small number of proteins expressed by advanced germ cells within the blood-testis barrier appeared in TIF. Finally, we show an 80% commonality between TIF proteins and blood plasma, providing a potential readily-accessible route by which changes in testicular protein markers, and thereby testicular function, could bemonitored in blood.

The utility of non-invasive protein biomarkers of spermatogenesis in the assessment of male fertility has long been suggested ([5], and reviewed [41]), but progress has been slow. With the advent of more powerful mass spectrometry-based methodologies, several groups have conducted extensive proteomic characterisation of whole mouse and human testes and isolated rodent germ cell types [42-44], allowing transcriptomic / proteomic comparisons to be made (e.g.[27, 44]). To address the aetiology of human infertility, comparative proteomic mapping of seminal plasma from fertile and infertile men has been investigated [13, 45], but

produced limited success in distinguishing phenotypes of non-obstructive azoospermia, possibly because <10% of seminal plasma is of testicular origin (reviewed in [45]).

To enable a more direct analysis of testicular fluid output, Chalmel et al examined the proteome of rat and ram STF collected at the rete testis, and found it to contain proteins preferentially secreted by Sertoli cells and germ cells [27]. However this fluid is difficult to access and only available in low volume from rodent models [27]. In contrast, TIF is easier to obtain [9] and studies have shown it contains proteins from blood-plasma as well as from Sertoli- and germ- cell sources (e.g. [5, 7]). Our analysis has extended these findings considerably by using mass spectrometry to identify 276 proteins in TIF, of which 64% are expressed by Sertoli cells and spermatogonia. Since these cells have free access to the interstitium, they are likely to have a major contribution to the protein composition of TIF. Our analysis also reveals that TIF contains some proteins enriched in mature germ cells such as round spermatids. Various precedents already exist for circulating proteins derived from meiotic and post-meiotic germ cells including the lactate dehydrogenase-C4 isoenzyme in control rats [7, 46], and *Fabp9* and *Ddx4* found in TIF in rats with compromised blood-testis barrier function [47]. We also noted proteins that were highly expressed in Sertoli cells and appeared only in TIF and not STF (*Ehd3*, *Fmod*, *Gnl3*, *Phgdh*), suggesting that they could be secreted in a basal direction. Directional secretion of Sertoli cell proteins is an important functional marker of mature Sertoli cell function [48], therefore it is feasible that the level of particular Sertoli cell proteins in TIF could reflect the presence of a functional seminiferous epithelium, with an intact blood-testis-barrier. These interpretations are subject to a number of untested assumptions, including that mRNA expression need not equate with protein secretion, and that the presence / absence of a signal by mass spectrometry may be an issue

related to detection sensitivity rather than tissue specificity. Our future studies will focus on identifying functional markers of spermatogenesis by comparing TIF from normal and various dysfunctional states, including the absence of mature germ cells, and disruption to Sertoli cell maturation and function.

TIF contains components derived from blood as a capillary filtrate, and TIF components can leave the testis via the intertubular lymphatic system [49]. In common with other interstitial fluids [50], our analysis found TIF contained various blood plasma proteins including albumin, complement factors, apolipoproteins, protease inhibitors and immunoglobulins. However access of these serum proteins into TIF is not passive as the testicular capillary endothelium is unfenestrated [51], hence entry occurs via facilitated diffusion and specific transcapillary transport mechanisms that remain poorly understood [8]. The simple, gentle method used to collect rat TIF [5, 7, 47] yielded a clear yellow liquid with virtually no red blood cells present, suggesting minimal contamination of TIF by blood.

In conclusion, we have identified 276 proteins in normal adult rat TIF, and by cross-matching with existing proteomic and mRNA expression databases from testicular cells and fluids, show that we can detect proteins contributed by the major cell types (Sertoli, Leydig, peritubular myoid, germ) in seminiferous tubules. Several proteins are highly enriched in specific cell types, and could potentially reflect spermatogenic function. Additionally TIF has only 36% similarity to STF, indicating that these fluids are quite different, but in contrast >80% of TIF proteins are also found in human plasma based on an *in silico* comparison, suggestive that ready exchange occurs between these fluids. This ‘proof of concept’ study has established that TIF protein analysis is feasible and able to reveal novel information about testicular function. We suggest that these results are an important starting point, however an

increased depth of proteomic information about TIF will be possible with more advanced mass spectroscopy techniques as recently demonstrated with other testicular fluid proteomes [27, 28]. We anticipate that this study will serve as an important resource for studies aimed at identifying signalling pathways between testicular cell types in the normal testis, and opens the way for investigative studies to assess the impact of changes in one cell type on the functions of others, such as germ cell loss on Sertoli cell health. Finally, as first predicted by Sharpe more than 20yrs ago [5], the proteomic monitoring of proteins in TIF and potentially serum from fertile and infertile men could provide a new investigative means to assess cellular and molecular events relevant to testicular disease and infertility.

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Disclosure statement

The authors have declared no conflict of interest.

Figure Legends

Figure 1. Organisation of the testis. The testis consists of multiple seminiferous tubules within which sperm are produced and leave via the rete testis, epididymis and vas deferens. A cross section of the testis shows two seminiferous tubules, comprised of germ cells at

multiple stages of development in close contact with somatic Sertoli cells, forming the seminiferous epithelium. Surrounding the tubules is a layer of peritubular myoid cells which have contractile properties. Outside of the tubules is the interstitium containing androgen-producing Leydig cells, macrophages, and blood and lymphatic vessels; collectively these cells and vessels are contained within a proteinaceous testicular interstitial fluid (TIF). Tight junctions between individual Sertoli cells form the blood-testis barrier, providing a seal which sequesters more advanced germ cell types in the inner, or adluminal, region away from earlier germ cell types and interstitial cells outside the barrier. Sertoli cells secrete many proteins in a bi-directional manner, either basally to contribute to TIF and potentially to the blood-stream, or via the Sertoli cell apex into the seminiferous tubule fluid which transports released mature spermatids to the epididymis. The presence of the blood-testis barrier prevents uncontrolled mixing between seminiferous tubule fluid and testicular interstitial fluid. Figure adapted from [52].

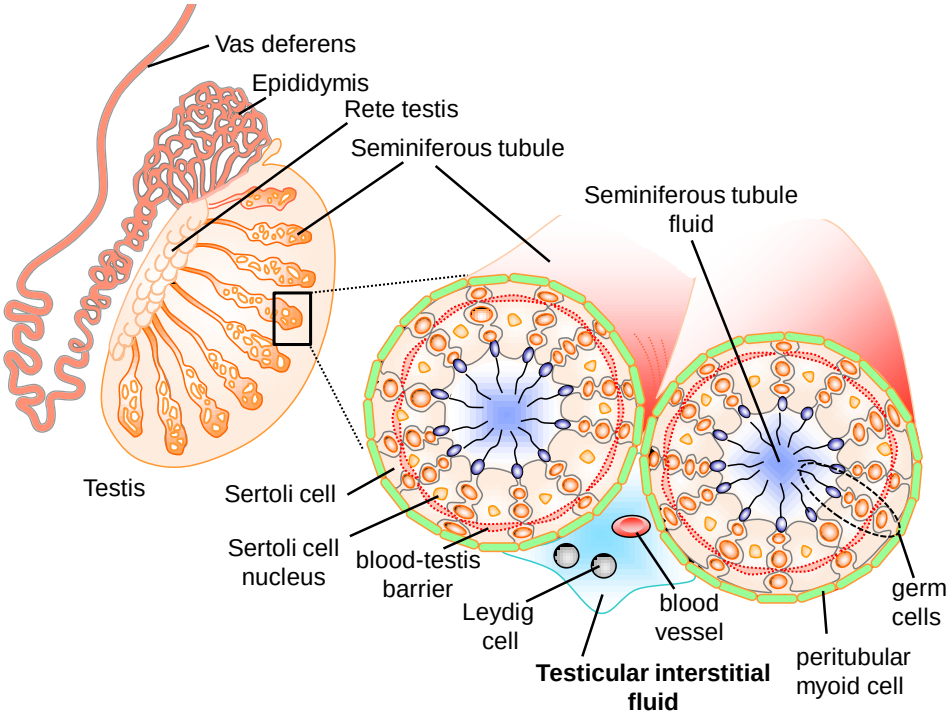


Figure 2. A) SDS-PAGE on 4-12% SDS gradient gel of (1) adult rat testicular interstitial fluid, (2) non-enriched TIF proteins, (3) enriched TIF proteins. ~250ng total protein was loaded under reducing conditions, and the gel stained with Sypro Ruby. Molecular weight markers ranged from 200 to 15 kDa. B) Comparison of proteomes from rat TIF, rat and ram seminiferous tubule fluid (STF), and human peritubular myoid cells (PTMC) (details of sources are included in legend to Table 1). Proteomes were compared based on common gene IDs with total gene numbers in each group indicated by *, and italicised figures represent %'s of the total TIF proteome.

Figure 2

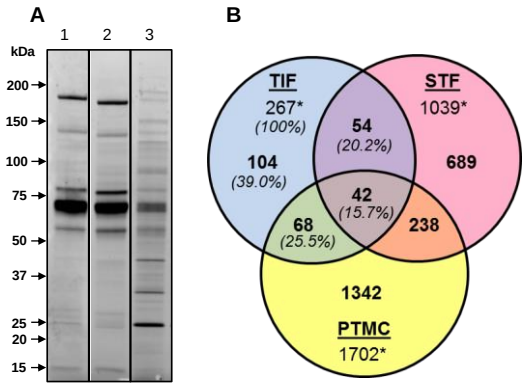
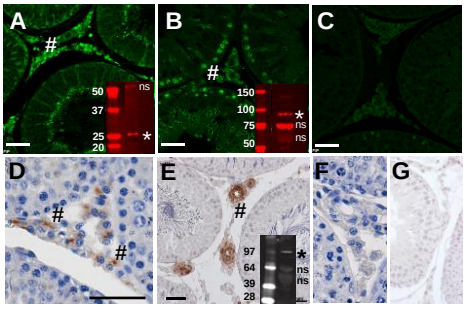


Figure 3. Immunohistochemistry and western blot of A) Lum (lumican), B) Msh2 (DNA mismatch repair protein Musashi 2), C) negative control = non-immune rabbit IgG at equivalent concentration to primary antibodies for panels A – B (details see Supplementary Table 1), D) INSL3 (Insulin-like 3), E) Actn4 (Alpha-actinin-4), F-G) negative controls = normal goat serum for panels D & E. Insets are western blots of rat TIF probed with the respective antibodies, # indicates positive staining in sections, * = specific band on westerns, ns = non-specific band. Bar = 50µm.

Figure 3



Tables

Table 1. Names and expression comparisons of top 30 proteins in adult rat testicular interstitial fluid based on MOWSE scores. The full table containing 276 proteins is shown in Supplementary Table 2.

							Comparison with proteomic data						Comparison with mRNA expression data				
							Protein is present (yes) or not found (-) in testicular fluid/cell proteomes. (TIF = testicular interstitial fluid, STF = seminiferous tubule fluid, PTMC = peritubular myoid cell)						Cell types within seminiferous tubules		Interstitial cell types		
Rank	Accession	Gene symbol	Protein	MOWSE Scores	# Peptides Identified	Sequence Coverage [%]	Rat TIF	Ram STF (1)	Rat STF (1)	Total PTMC (2)	Secreted PTMC (3)	Human plasma proteome (4)	Rat epithelial mRNA expression pattern: Johnston 2008 (5) or Chalmel 2007 (6)[#]	Cell grouping with preferential expression. basal = (SC & Sg), adluminal = (PS & rST) (7)	Rat Leydig cell specific (8)*	Rat Leydig cell specific (9)*	Mouse Leydig cell specific (10)*
1	gi: 158138561	C3	complement C3 [Rattus norvegicus]	4179	43	42.9	yes	yes	yes	yes	yes	yes	NE[@]		-*	-	yes
2	gi: 8392909	Apoa4	apolipoprotein A-IV precursor	3030	29	74.2	yes	yes	-	-	-	yes	NE		-	-	-

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			[Rattus norvegicus]														
3	gi: 77861917	Cfh	complement factor H [Rattus norvegicus]	2631	30	41.8	yes	yes	-	yes	yes	yes	Sg>>SC&PS& rST	basal	-	-	-
4	CO4_RAT	C4	Complement C4 OS=Rattus norvegicus	1959	21	27.5	yes	-	yes	-	-	yes	no data[#]		-	-	-
5	APOA1_RAT	Apoa1	Apolipoprotein A-I OS=Rattus norvegicus	1906	21	64.5	yes	yes	-	-	-	yes	NE		-	-	-
6	gi: 149015982	Fn1	fibronectin 1, isoform CRA_e [Rattus norvegicus]	1874	22	22.4	yes	-	-	yes	yes	yes	Sg>>SC>>PS& rST	basal	-	-	yes
7	FIBB_RAT	Fgb	Fibrinogen beta chain OS=Rattus norvegicus	1852	23	51.8	yes	yes	yes	-	-	yes	NE		-	-	-
8	gi: 158138568	Alb	albumin [Rattus norvegicus]	1852	19	42.6	yes	yes	yes	-	-	yes	NE		-	-	-
9	gi: 149048530	Cp	ceruloplasmin, isoform CRA_a [Rattus norvegicus]	1777	17	25.7	yes	-	yes	-	-	yes	Sg>SC>>rST& PS	basal	-	-	yes
10	VTDB_RAT	Gc	Vitamin D-binding protein OS=Rattus norvegicus	1569	16	47.9	yes	yes	yes	-	-	yes	NE		-	-	-
11	gi: 56797757	Fga	fibrinogen alpha chain isoform 1 [Rattus norvegicus]	1370	12	29.8	yes	yes	-	-	-	yes	NE		-	-	-
12	gi: 126722991	Itih4	inter-alpha-inhibitor H4 heavy chain [Rattus norvegicus]	1335	12	26.4	yes	yes	-	-	-	yes	NE (interstitial)		-	-	-
13	PLMN_RAT	Plg	Plasminogen OS=Rattus norvegicus	1078	16	40.1	yes	yes	yes	-	-	yes	NE		-	-	-
14	gi: 83816939	A1i3	alpha-1-inhibitor 3 precursor [Rattus norvegicus]	1050	13	20.9	yes	-	-	-	-	-	NE		-	-	-

15	gi: 61556986	Tf	transferrin [Rattus norvegicus]	1005	11	30.1	yes	yes	yes	-	-	yes	SC>PS&rST>> Sg[#]		-	-	-
16	gi: 194474004	Col14a1	collagen alpha-1(XIV) chain [Rattus norvegicus]	1003	9	12.3	yes	-	-	yes	yes	yes	Sg>rST&PS>SC		-	-	-
17	TBA1B_RAT	Tuba1b	Tubulin alpha-1B chain OS=Rattus norvegicus	1001	10	46.6	yes	-	yes	yes	-	yes	SC&Sg>rST>> PS[#]	basal	-	-	-
18	gi: 119959830	Actb	beta-actin [Rattus norvegicus]	999	8	48.4	yes	-	yes	-	-	yes	SC&Sg&rST&PS		-	-	-
19	gi: 38328248	Tuba1a	Tubulin, alpha 1A [Rattus norvegicus]	980	9	43.5	yes	-	-	yes	-	yes	SC&Sg>rST>> PS[#]	basal	-	-	-
20	gi: 149038928	Gsn	gelsolin, isoform CRA_a [Rattus norvegicus]	960	8	25.8	yes	yes	yes	yes	-	yes	Sg>SC>rST&PS	basal	-	-	yes
21	THRB_RAT	F2	Prothrombin OS=Rattus norvegicus	935	11	29.0	yes	yes	-	-	-	yes	NE		-	-	-
22	gi: 8393418	Gapdh	glyceraldehyde-3-phosphate dehydrogenase [Rattus norvegicus]	930	11	43.5	yes	yes	yes	yes	-	yes	SC&Sg>>PS& rST[#]	basal	-	-	yes
23	gi: 122065184	Fgg	RecName: Full=Fibrinogen gamma chain; Flags: Precursor	926	10	40.4	yes	-	yes	-	-	yes	PS>rST&Sg&SC	adluminal	-	-	-
24	gi: 109472532	A2m	PREDICTED: alpha-2-macroglobulin [Rattus norvegicus]	899	9	15.0	yes	yes	yes	yes	-	yes	SC>>rST&PS& Sg		-	-	-
25	gi: 162287178	Vtn	vitronectin [Rattus norvegicus]	890	8	23.2	yes	-	-	-	-	yes	SC&Sg>>rST& PS	basal	-	-	-
26	TBA1C_RAT	Tuba1c	Tubulin alpha-1C chain OS=Rattus norvegicus	889	9	40.3	yes	-	yes	-	-	yes	SC&Sg>rST>> PS#		-	-	-

27	MUG1_RAT	Mug1	Murinoglobulin-1 OS=Rattus norvegicus	888	11	13.4	yes	-	yes	-	-	-	NE[#]		-	-	-
28	ITIH3_RAT	Itih3	Inter-alpha-trypsin inhibitor heavy chain H3	868	7	25.6	yes	-	-	-	-	yes	**rST**>>PS&SC> Sg	adluminal	-	-	-
29	SPA3K_RAT	Serpina3k	Serine protease inhibitor A3K OS=Rattus norvegicus	781	10	48.3	yes	-	yes	-	-	-	NE		-	-	yes
30	gi: 11066005	Hrg	histidine-rich glycoprotein [Rattus norvegicus]	774	10	20.8	yes	-	-	-	-	yes	rST&PS[#]	adluminal	-	-	-

Explanatory notes

(1) By comparison with Supplementary Table S1[27]. (2) By comparison with Supplementary Table 1 [28]. (3) Secreted proteins by comparison with Supplementary Table 5 [28]. (4) Data from Human Plasma Protein Database (http://www.plasmaproteomedatabase.org). (5) By comparison with data from [31] (6) By comparison with [30] and searchable online (http://www.germonline.org/index.html). '#' indicates that data is from [30]. If no symbol, data is from [31].

'@ NE': not expressed or below detection limits. 'No data': no probeset that matches the gene symbol. 'Sg': spermatogonia, 'SC': Sertoli cell, 'PS': pachytene spermatocyte, 'rST': round spermatid. '>' indicates expression greater than 2-fold, but less than 4-fold different to the next cell type. '>>' indicates expression greater than 4-fold compared with next cell type. **Bold text** indicates 10-fold higher expression than next cell type. '&' indicates expression differences between cell types is less than 2-fold. 'NE (interstitial)' indicates a positive signal in seminiferous tubule preps, but not in isolated germ or Sertoli cells, hence signal is likely interstitial. (7) Where possible, cell expression has been grouped as either basal (Sertoli cells, spermatogonia) or adluminal (pachytene spermatocyte, round spermatid) to reflect the presence of the blood-testis barrier which separates these two cell groupings. Where this pattern was not observed (ie: equal expression across all cell types) no grouping was possible. (8) By comparison with Supplementary Table 2, [32]. (9) by comparison with Supplemental Table S3 [33] (10) By comparison with Dataset S2 Leydig cell enriched genes, [34]. '*' The Leydig cell data in (8), (9) and (10) is processed and only records genes judged by the authors to be Leydig cell-specific. The (-) symbol indicates no data available, but should not be interpreted as a lack of expression.

Table 2. Functional annotation clustering for the Gene Ontology term 'Biological Process' using all rat TIF proteins. A more detailed version of this table which includes gene names for each Biological Process is shown in Supplementary Table 4. Clusters with an FDR >0.01 were rejected from this analysis.

Annotation Cluster	Cluster Name	Enrichment score	GO Biological Process	Fold Enrichment	P Value	FDR
1	Coagulation	11.77	GO:0050817~coagulation	13.02	5.14E-12	8.88E-09
2	Acute inflammatory response	7.41	GO:0006958~complement activation, classical pathway	28.65	1.72E-11	2.98E-08
			GO:0002541~activation of plasma proteins involved in acute inflammatory response	21.91	2.16E-13	3.72E-10
			GO:0051605~protein maturation by peptide bond cleavage	12.74	1.15E-12	1.99E-09
			GO:0002526~acute inflammatory response	16.56	9.67E-24	1.67E-20
			GO:0016064~immunoglobulin mediated immune response	13.13	7.41E-09	1.28E-05
			GO:0002443~leukocyte mediated immunity	9.93	5.50E-09	9.49E-06
3	Assembly of complex proteins	5.81	GO:0051258~protein polymerization	19.10	1.26E-09	2.18E-06
			GO:0043623~cellular protein complex assembly	6.98	9.28E-08	1.60E-04
			GO:0043933~macromolecular complex subunit organization	2.89	7.52E-07	1.30E-03
4	Nutritional response	5.46	GO:0007584~response to nutrient	5.01	1.07E-07	1.85E-04

	pathway		GO:0009991~response to extracellular stimulus	4.17	6.40E-08	1.11E-04
5	Peptide bond cleavage	5.11	GO:0006508~proteolysis	2.88	4.57E-09	7.89E-06
6	Negative regulation of clotting	4.08	GO:0050819~negative regulation of coagulation	24.13	1.82E-08	3.14E-05
			GO:0032101~regulation of response to external stimulus	5.34	7.86E-07	1.36E-03
7	Hormone signalling	3.86	GO:0009719~response to endogenous stimulus	2.80	2.19E-06	3.79E-03
8	Protein polymer biosynthesis	3.6	GO:0051258~protein polymerization	19.10	1.26E-09	2.18E-06
9	Regulation of cellular protein metabolism	3.12	GO:0032268~regulation of cellular protein metabolic process	3.31	9.11E-07	1.57E-03
10	Regulation of cholesterol transport	2.85	GO:0032371~regulation of sterol transport	23.60	2.70E-07	4.66E-04
11	Cell cycle	2.85	GO:0022402~cell cycle process	3.38	3.84E-06	6.64E-03

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